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HSP-72 synthesis is promoted by increase in $[Ca^{2+}]_i$ or activation of G proteins but not pH_i or cAMP

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pertussis toxin; cholera toxin; 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid

THE PRESENCE OF STRESSORS can alter epithelial function. A significant stressor is heat shock, which can occur during a fever caused by an infection or by environmental heat exposure. It is known that hyperthermia induces the synthesis of heat-shock proteins (51) with molecular masses ranging from 28 to 174 kDa (HSP-28 to HSP-174) that can be found in bacteria, yeast, *Drosophila*, and other organisms, including humans (2, 32, 47).

In intact animals, heat-shock proteins are induced by fever (6), ischemia (6, 12, 14, 18, 28, 38, 54), hyperthermia (1, 9, 11), and exercise (44). Heat-shock proteins induced in vitro by hyperthermia or other means have been found in many different types of cells such as mammary glands (7), fibroblasts (53), monocytes (20, 40), Chinese hamster ovary cells (31), GH_3 pituitary cells (5), GC pituitary cells (48), and cultured cerebellar astrocytes and granule cells (33).

Heat-shock proteins are highly conserved evolutionarily. It is assumed that they serve an important protective function in response to stress. The HSP-70 family is known to reduce thermosensitivity and increase thermotolerance, which enhances cell survival from thermal injury, a phenomenon readily observed in cells pretreated with heat (19, 43, 48, 57). An inducible form of the HSP-70 family, HSP-72, has been studied extensively. HSP-72 has been shown to protect cells not only from heat but also from other forms of injury, such as the toxicity of hydrogen peroxide in human monocytes (40), light damage to rat retina (1), ethanol-induced damage in cultured guinea pig gastric mucosal cells (37), and ischemia-reperfusion damage in rabbit heart (8).

In addition to inducing HSP-72, hyperthermia also causes an intracellular acidification (26) and increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and adenosine 3′,5′-cyclic monophosphate (cAMP) (23, 27). It is known that changes in intracellular pH (pH_i) alter cell growth and differentiation, and changes in $[Ca^{2+}]_i$ alter metabolic systems. In this study, we characterized the relationship between HSP-72, pH_i, $[Ca^{2+}]_i$, G proteins, and cAMP in human epidermoid A-431 cells. We found that the synthesis of HSP-72 and its mRNA depended on an increase in $[Ca^{2+}]_i$ and was potentiated by G proteins but was not related to a heat-induced intracellular acidification or an increase in cellular cAMP.

MATERIALS AND METHODS

Cell culture. Human epidermoid carcinoma A-431 cells (American Type Culture Collection, Rockville, MD) were grown in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA), containing Dulbecco's modified Eagle's medium with 0.03% glutamine, 4.5 g/l glucose, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10% fetal bovine serum, penicillin (50 μg/ml), and streptomycin (50 U/ml; GIBCO-BRL, Gaithersburg, MD), and were incubated in a 5% CO_2 atmosphere at 37°C. Cells were fed every 3–4 days. Cells from passages 28–50 were used for the experiments.

Gel electrophoresis. Heat treatment was performed by adding culture medium at the specified temperature to a culture flask of confluent cells and placing the flask into a water bath at the same temperature for the specified time. At the end of heat exposure, the medium was replaced with fresh medium at 37°C, and the flask was returned to the incubator at 37°C. After the specified time, cells were removed from the flask and

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were centrifuged at 750 *g* for 10 min at 4°C, and the pellet was lysed in buffer containing 1% sodium dodecyl sulfate (SDS) plus 1% mercaptoethanol. Aliquots corresponding to 15 μg of protein were resolved on SDS-polyacrylamide slab gels (Novex precast 10% gel, San Diego, CA) according to the method of Laemmli (30). Protein bands were then quantified by laser densitometry (Molecular Dynamics model 300B, San Diego, CA). The HSP-72 band was normalized to the actin band to correct for variations in the amount of protein loaded onto the gel. Heat stress does not affect actin synthesis (31).

For experiments requiring radioactive labeling, cells were incubated in medium containing [^{35}S]methionine for 1 h before the cells were removed for determination of heat-shock proteins. Equal counts per minute of each sample were loaded onto the gel. Radioactivity was quantified by laser densitometry of the autoradiograph (Molecular Dynamics).

Dot blot. A cDNA probe for HSP-70 mRNA was obtained by restriction digest of a pAT153 vector (American Type Culture Collection), containing the pH 2.3 cDNA clone for human (chromosome 6) HSP-70 (17). *Hind* III/*Bam*H I restriction digests were separated into 2.3- and 3.3-kb fragments on 1% low-melting temperature agarose gels that contained ethidium bromide. The 2.3-kb fragment, which is the HSP-70 cDNA insert, was isolated and labeled with ^{32}P.

Total RNA was isolated from cell extracts by the method of White and Bancraft (56). Briefly, monolayers of confluent cells (5×10^6 cells) were scraped from culture flasks and resuspended in a solution containing 45 μl ice-cold TE buffer (10 mM tris(hydroxymethyl)aminomethane (Tris)·HCl and 1 mM EDTA, pH 7.4) and 5 μl 5% Nonidet P-40 (NP-40). The suspension was placed on ice for 5 min, another 5 μl 5% NP-40 was added, and the incubation on ice was continued for an additional 5 min. The mixture was centrifuged at 1,500 *g* for 2 min. Thirty microliters of 20× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 20 μl 37% formaldehyde were added to 50 μl of the supernatant, and the mixture was incubated at 60°C for 15 min. The sample was stored at −70°C until used.

RNA (25 μl) was loaded into a microsample filtration manifold (Schleicher and Schuell, Keene, NH). A cDNA probe for 18S rRNA was used to correct for small differences in RNA loading. Membranes were prehybridized overnight at 55°C with 100 μg/ml salmon testes DNA (Sigma, St. Louis, MO) in a hybridization buffer containing 6× SSC, 5× Denhardt's reagent, and 0.5% SDS to prevent nonspecific binding. Membranes were then hybridized overnight at 55°C in the same hybridization buffer with the radiolabeled cDNA probe for HSP-70 mRNA and washed sequentially in 0.1% SDS plus 6× SSC, 2× SSC, and 0.1× SSC at 65°C. Hybridized membranes were autoradiographed (X-Omat film, Kodak, Rochester, NY) with an intensifying screen at −70°C. Radioactivity was quantified by direct radiographic imaging of the membrane (Ambis, San Diego, CA).

Preparation of nuclear extract and electrophoretic mobility-shift assays. Confluent cells in 150-cm^2 flasks were trypsinized and spun at 750 *g* for 5 min. The cell pellet was washed in a hypotonic buffer [containing in mM: 10 HEPES, 1.5 $MgCl_2$, 10 KCl, 0.2 phenylmethylsulfonyl fluoride (PMSF), and 0.5 1,4-dithiothreitol (DTT), pH 7.9 at 4°C] and spun again at 750 *g* for 5 min. The cell pellet was then resuspended in hypotonic buffer and left on ice for 10 min. Cells in suspension were transferred to glass Dounce homogenizer to homogenize cells. The homogenate was centrifuged for 15 min at 3,300 *g*. The pellet that contained the nuclei was resuspended in low-salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT, pH 7.9 at 4°C). The mixture was gently stirred at 4°C for 30 min and centrifuged at 25,000 *g* for 30 min. The supernatant that contained the nuclear proteins was placed in dialysis tubing (mol wt cutoff: 6–8 kDa) and dialyzed against buffer (20 mM HEPES, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT, pH = 7.9, 4°C) for 1 h. The supernatant was frozen at −70°C until use. Various amounts of nuclear proteins were incubated with ^{32}P-labeled 5′ end-labeled heat-shock element (HSE; GIBCO-BRL) at 25°C for 30 min in binding buffer (5% glycerol, 10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 40 mM KCl, and 0.5 mM DTT), containing 2 mg/ml polydeoxyinosinic-deoxycytidylic acid. The samples were loaded to nondenaturized 5% Tris-borate EDTA (TBE) Bio-Rad mini-protein II ready gels in 1× TBE buffer. The gel was autoradiographed with an intensifying screen at −70°C. Radioactivity was quantified by laser densitometry of the autoradiograph.

Western blot. SDS-polyacrylamide gels were run as described in *Gel electrophoresis*. Protein was blotted onto a nitrocellulose membrane (type NC, 0.45 μm, Schleicher and Schuell), using a Novex blotting apparatus and the manufacturer's protocol. The blot was processed according to a method described by Sarge et al. (45). Briefly, after the nitrocellulose was blocked by incubation for 90 min at room temperature in phosphate-buffered saline (PBS) containing 2.5% nonfat dried milk, the blot was incubated for 60 min at room temperature with mouse monoclonal antibody directed against HSP-72 (Amersham, Arlington Heights, IL) at a 500× dilution in PBS-5% bovine serum albumin (BSA) containing 0.1% thimerosal. The blot was then washed 3 times (10 min each) in PBS-0.1% Tween 20 before incubating the blot for 60 min at room temperature with a 1,000× dilution of rabbit anti-mouse immunoglobulin G peroxidase conjugate (Amersham) in PBS-1% gelatin. The blot was washed six times (5 min each) in PBS-0.1% Tween 20 before detection of the peroxidase activity, using the substrate 4-chloro-1-naphthol (Sigma).

pH_i measurements. Confluent monolayers of A-431 cells on coverslips were placed in Na^+ Hanks' solution (containing in mM: 145 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES, pH 7.40), containing 5 mM glucose, 0.2 % albumin, and 5 μM 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-acetoxymethyl ester (AM) and incubated for 30 min at 37°C. Cells were then washed with Na^+ Hanks' solution before fluorescence measurements. The rate of leakage of this fluorescent dye at 37 and 45°C and the method used to determine pH_i in A-431 cells have been published previously (26). To acid load the cells, cells were incubated in Na^+ Hanks' solution, containing 30 mM NH_4Cl for 15 min then incubated in Na^+-free Hanks' solution (Na^+ replaced by equimolar *N*-methyl-D-glucamine) for 2 min. To alkalinize cells, cells were exposed to Na^+ Hanks' solution containing 30 mM NH_4Cl for 15 min. The pH_i was verified with BCECF measurement.

$[Ca^{2+}]_i$ measurements. Confluent monolayers of cells were loaded with 5 μM fura 2-AM plus 0.2% pluronic F-127 at 37°C for 60 min. Cells were then washed with Na^+ Hanks' solution before fluorescence measurements. The leakage rate of this fluorescent dye at 37 and 45°C and the method to determine $[Ca^{2+}]_i$ have been described previously (22).

Statistical analysis. All data are expressed as means ± SE. Analysis of variance, Student's *t* test, Studentized range test, and Bonferroni's inequality were used for comparison of groups (49).

Chemicals. Chemicals used in this study were BSA, *N*-methyl-D-glucamine, forskolin (FSK), 8-bromo-cAMP, 3-isobutyl-1-methylxanthine (IBMX), pertussis toxin (PTX), cholera toxin (CTX), A-23187 (Sigma), 1-[6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1H-pyrole-2,5-dione (U-

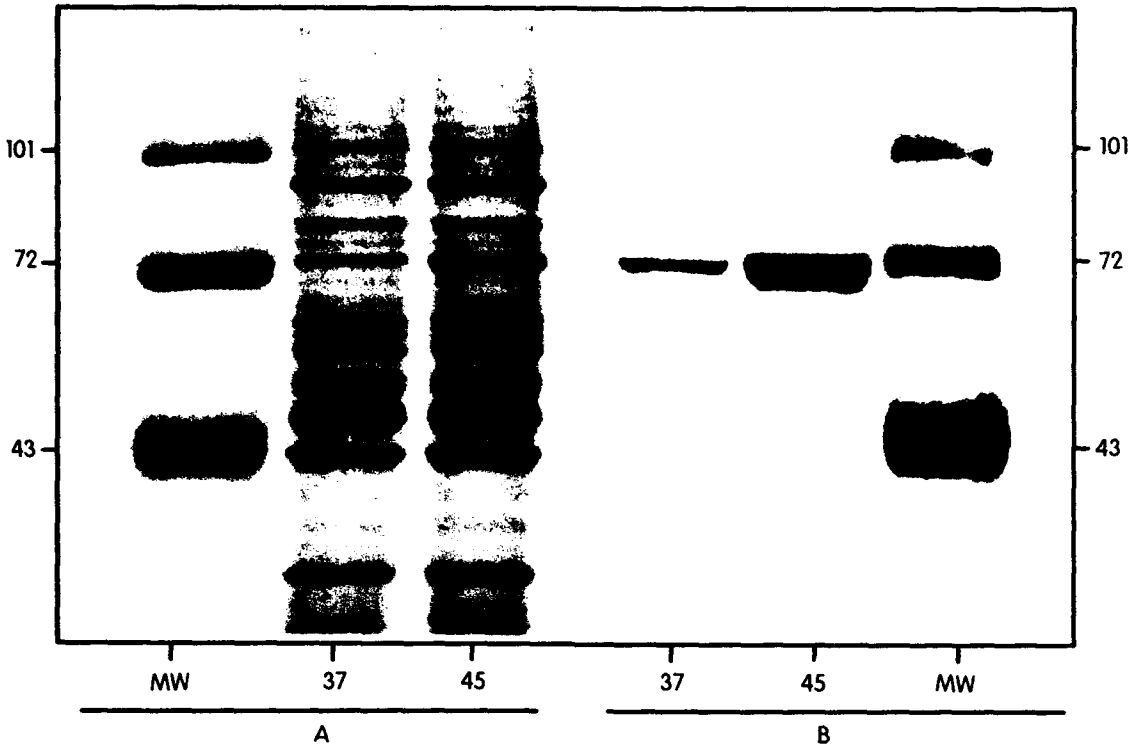

Fig. 1. Identification of a 72-kDa heat-shock protein (HSP-72). Cells were heated at 45°C for 10 min in Na^+ Hanks' solution, then returned to 37°C for 8 h before they were prepared for electrophoresis. Proteins were separated in SDS-polyacrylamide gels and either stained with Coomassie blue (*A*) or transferred to a nitrocellulose membrane that was used to confirm the identity of HSP-72 by Western immunoblotting (*B*). Samples from unheated (37) and heated (45) cells are shown. Prestained molecular weight (MW) markers (GIBCO-BRL) are (from top to bottom) phosphorylase B, bovine serum albumin, and ovalbumin, with their apparent molecular masses indicated in kDa. The prominent band induced in heated cells at the 72-kDa position is HSP-72.

73122; provided by Upjohn, Kalamazoo, MI), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem, La Jolla, Ca), ionomycin, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), fura 2-AM, BAPTA-AM, and BCECF-AM (Molecular Probes, Eugene, OR).

RESULTS

Effect of heat on HSP-72. Exposing A-431 cells to heat at 45°C for 10 min and then returning them to 37°C for 8 h induced HSP-72 (Fig. 1*A*). Western blotting verified the identity of HSP-72 (Fig. 1*B*). The increase in HSP-72 depended on the temperature of heating. Heating at 39°C for 10 min followed by an incubation at 37°C for 8 h induced HSP-72 slightly, but heating at 41°C for 10 min resulted in a significant increase in HSP-72 (Fig. 2*A*). Exposure to 45°C for 10 min produced maximal induction. Heating to 48°C resulted in less HSP-72 induction, perhaps related to a small loss of viability after the heat shock (26). The amount of HSP-72 produced by the cells was also dependent on the duration of heating. HSP-72 increased in cells after only a 1-min exposure to 45°C (as measured after a 5-h incubation at 37°C). Induction reached a maximum in cells heated for 10 min (Fig. 2*B*). A longer exposure to heat did not further increase HSP-72.

Likewise, a second heat exposure at times before the maximal induction of HSP-72 at 8 h did not affect HSP-72 induction (Fig. 3). For these experiments, cells received a second heat shock 2, 4, or 6 h after the first heating, and HSP-72 synthesis was measured 8 h after the first heating. The level of HSP-72 in the cell after the second heating was similar to that measured after one heating only, suggesting that the cellular mechanisms involved in the initial response to heat somehow prevent a similar response when the cells are heated a second time.

The size of the heat-induced increase in HSP-72 depended also on the period of time that cells were incubated at 37°C after heating. Figure 4*A* shows that the HSP-72 was first observed 2 h after heat shock. Maximal production was at 8 h, and elevated levels could still be observed at 4 days, the longest interval tested.

Effect of heat on HSP-72 transcription and translation. We performed a series of experiments to determine whether the promotion of HSP-72 induction by heat occurred at the transcriptional or the translational level. HSP-72 mRNA was measured, using human HSP-70 cDNA to probe for the inducible HSP-72 mRNA. HSP-72 mRNA levels increased immediately after heating, reached a maximum after 2 h, and returned to baseline within 8 h (Fig. 4*B*). This mRNA time course correlated with the time course for protein production. In another experiment, cells were treated with actinomycin D (a

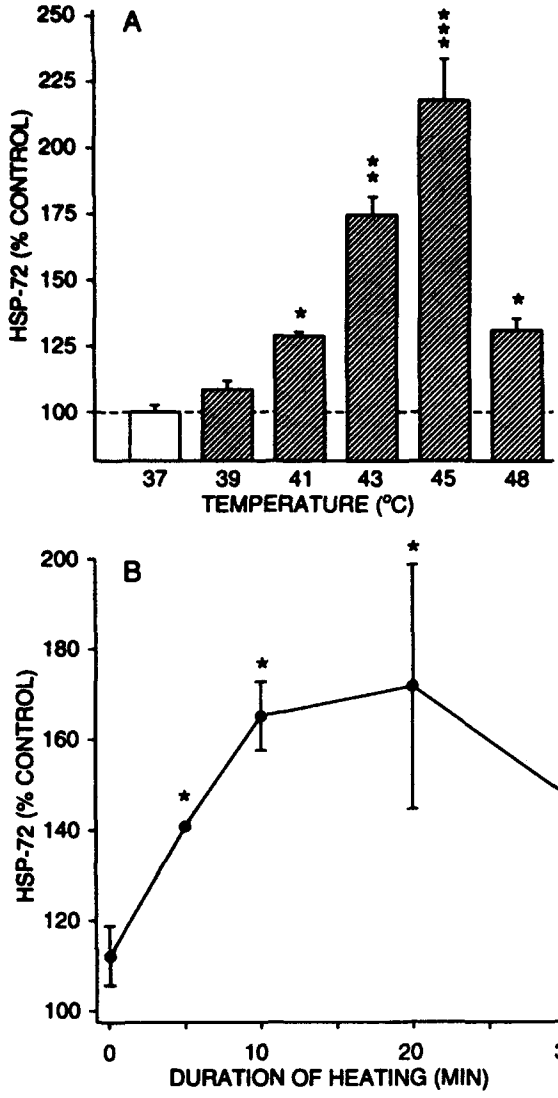

Fig. 2. *A*: effect of temperature on HSP-72 induction. Cells were heated at specified temperature for 10 min then returned to 37°C for 8 h ($n = 5$). *$P < 0.05$ vs. 37 and 39°C, **$P < 0.05$ vs. 37, 39, 41, and 48°C, ***$P < 0.05$ vs. 37, 39, 41, 43, and 48°C; one-way analysis of variance (ANOVA) and Studentized range test. Control, open bar; experimental, hatched bars. *B*: effect of heating duration at 45°C on HSP-72 induction. Cells were exposed to 45°C for 1, 5, 10, 20, or 30 min then returned to 37°C for 5 h ($n = 3$). Error bars are SE (smaller than symbol for 5 min). *$P < 0.05$ vs. controls, Student's t test.

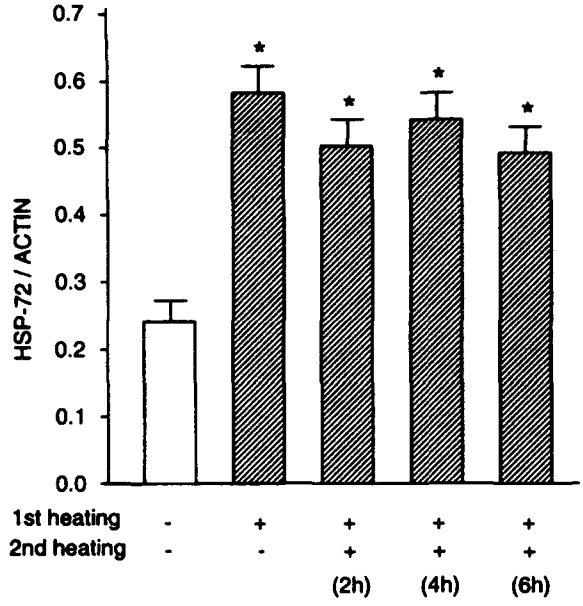

Fig. 3. Effect of a 2nd heating on HSP-72 induction. Cells were exposed to 45°C for 10 min (1st heating). Cells then received a 2nd heating (45°C, 10 min) 2, 4, or 6 h after the 1st heating. HSP-72 was determined 8 h after 1st heating ($n = 8$). *$P < 0.05$ vs. controls, 2-way ANOVA and Studentized range test. Control, open bar; experimental, hatched bars.

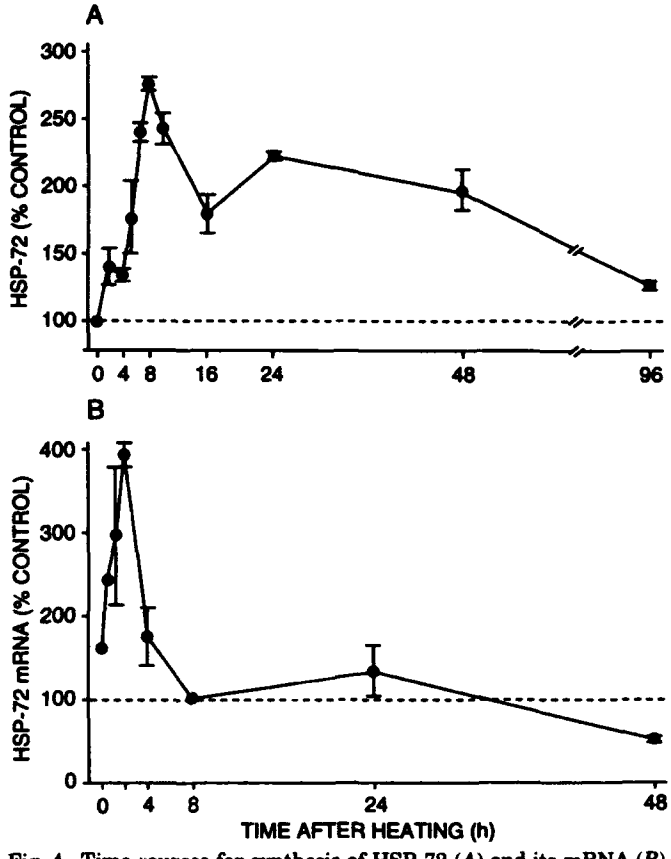

Fig. 4. Time courses for synthesis of HSP-72 (*A*) and its mRNA (*B*). Cells were exposed to 45°C for 10 min, then returned to 37°C for various periods of time before HSP-72 or mRNA was extracted ($n = 4$). Error bars are SE; baseline is 100% as marked.

transcription inhibitor) for 10 min before heating. Figure 5 shows that treatment with the drug completely prevented HSP-72 synthesis. The requirement for transcription after heating indicates that a heat-induced promotion of HSP-72 transcription is required for HSP-72 induction.

Figure 5 also demonstrates that treatment with cycloheximide (a protein synthesis inhibitor) 10 min before heating completely blocked the increase in HSP-72 induced by heat. This shows that the elevated levels of HSP-72 after heating are the result of new synthesis.

HSP-72 remained above the basal level 8 h after heating even though HSP-72 mRNA had returned to

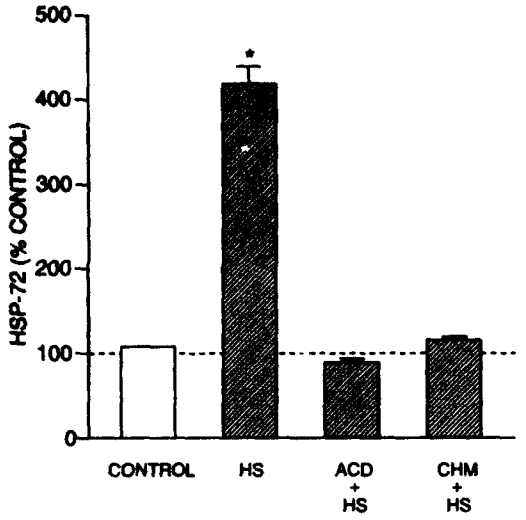

Fig. 5. Inhibition of HSP-72 by actinomycin D (ACD) and cycloheximide (CHM). Cells were treated with ACD (5 μg/ml) or CHM (60 μg/ml) 10 min before and during heating [heat shock (HS), 45°C, 10 min]. Cells were returned to 37°C for 8 h before determination of HSP-72 ($n = 6$). *$P < 0.05$ vs. controls, Student's t test.

baseline (Fig. 4A), which suggested either that HSP-72 is degraded slowly or that heat induces a persistent increase in protein translation activity. We conducted two experiments to determine if heat increased translational activity. In one experiment, cells were exposed to cycloheximide (60 μg/ml, 10 min) 8 h after heating, and HSP-72 was measured 24 h after heat exposure. This cycloheximide treatment affected unheated cells to a certain extent, because unheated cells treated with cycloheximide demonstrated a level of HSP-72 that was 39 ± 9% ($n = 4, P < 0.05$) lower than that measured in untreated cells. Cycloheximide treatment of heated cells resulted in a level of HSP-72 that was no different from that in cells heated without cycloheximide (heat alone: 239 ± 9%; cycloheximide + heat: 250 ± 29%, $n = 4, P > 0.05$). In another test of translation activity after heating, cells were pulse labeled with [^{35}S]methionine for 60 min 24 h after heating to measure the rates of protein synthesis at that time in heated and unheated cells. The incorporation of [^{35}S]methionine into HSP-72 was not different than that measured in unheated cells. These results reinforce the view that an ongoing increase in translational activity does not account for the high levels of HSP-72 levels measured up to 4 days after heating. The high levels of HSP-72 found in the cell are apparently due to translation of the early increased amount of HSP-72 mRNA stimulated by heating. The persistence of high levels of HSP-72 in cells is apparently due to the protein's low rate of degradation (half-life of ~48 h).

Because the maximal increase in HSP-72 occurred after a 10-min heating at 45°C followed by an 8-h incubation at 37°C, we used this protocol for the remainder of the experiments described in this study.

Effect of changes in pH_i on HSP-72. Because hyperthermia acidifies cells (26), it is plausible that the decrease in pH_i alone mediates the increase in HSP-72 levels measured in heated cells. To investigate the role of pH in this process, the pH_i of A-431 cells was adjusted to 6.9 or 7.8 (normal pH_i is 7.23 ± 0.02, see Ref. 26), and HSP-72 levels were determined. Intracellular acidification (pH_i 6.9) was obtained by prepulsing cells with 30 mM NH_4Cl for 15 min then exposing them to Na^+-free Hanks' solution (with Na^+ replaced by equimolar *N*-methyl-D-glucamine). Intracellular alkalinization (pH_i 7.8) was obtained by exposure to 30 mM NH_4Cl alone. A change in pH_i stimulated in unheated cells had no effect on levels of HSP-72 measured in unheated cells (HSP-72/actin at pH_i 6.9, 0.32 ± 0.03; pH_i 7.25, 0.34 ± 0.04; pH_i 7.8, 0.31 ± 0.04; $n = 12–16, P > 0.05$). But, in heated cells, changes in pH_i altered the amount of HSP-72 production. Intracellular alkalinization potentiated the production of the heat-induced HSP-72, whereas intracellular acidification attenuated it (pH 6.9, 170 ± 3%; pH 7.25, 223 ± 6%; pH 7.8, 262 ± 12%; $n = 7, P < 0.05$). However, that an induced acidification decreased, not increased, HSP-72 levels shows that a heat-induced acidification cannot, by itself, stimulate the HSP-72 induction measured in heated cells. We did not use nigericin (3 μM) and valinomycin (3 μM) in K^+ Hanks' solution to adjust pH_i in these experiments because treatment with these drugs during heating inhibited the HSP-72 induction (data not shown).

Effect of cAMP and G proteins on the heat-induced HSP-72. Because heat shock (45°C, 10 min) induces an increase in cellular cAMP levels of ~40% (27), it is possible that an increase in cAMP is a signal for the heat-induced HSP-72 production. If an increase in cellular cAMP induces HSP-72 production, then treatment with 1 mM IBMX (phosphodiesterase inhibitor), 1 mM 8-bromo-cAMP, 150 μM FSK (an adenylate cyclase stimulator), or 10 μM H-89 (protein kinase A inhibitor) should also elevate HSP-72. We determined that none of these treatments changed the basal level of HSP-72 in unheated cells (data not shown), suggesting that the heat-induced increase in cAMP is not related to HSP-72 induction. In heated cells, the degree of HSP-72 induction in cells treated with IBMX, 8-bromo-cAMP, or H-89 was not different from that measured in untreated cells.

FSK, however, enhanced heat-induced HSP-72 synthesis (Fig. 6A). We know that FSK increases cAMP by stimulating adenylate cyclase activity and promoting the interaction between adenylate cyclase and G proteins (27). We also know that heat shock stimulates G proteins in A-431 cells (24, 27). To determine whether G proteins are involved in HSP-72 induction, cells were pretreated with PTX (30 ng/ml, 24 h) or CTX (1 μg/ml, 1 h). Neither toxin changed the level of HSP-72 in unheated cells, but both potentiated HSP-72 mRNA and HSP-72 synthesis in heated cells (Fig. 6, *B* and *C*). Because both toxins can increase inositol trisphosphates (24), it was possible that inositol trisphosphate played a role in this potentiation. To test this possibility, cells were treated with 1 μM U-73122, an inhibitor for phospholipase C-mediated processes, including inositol trisphosphate production. U-73122 treatment reduced the levels of heat-induced HSP-72 (heated: 238 ± 17%, U-73122 + heated: 124 ± 8%; $n = 3–10, P < 0.05$).

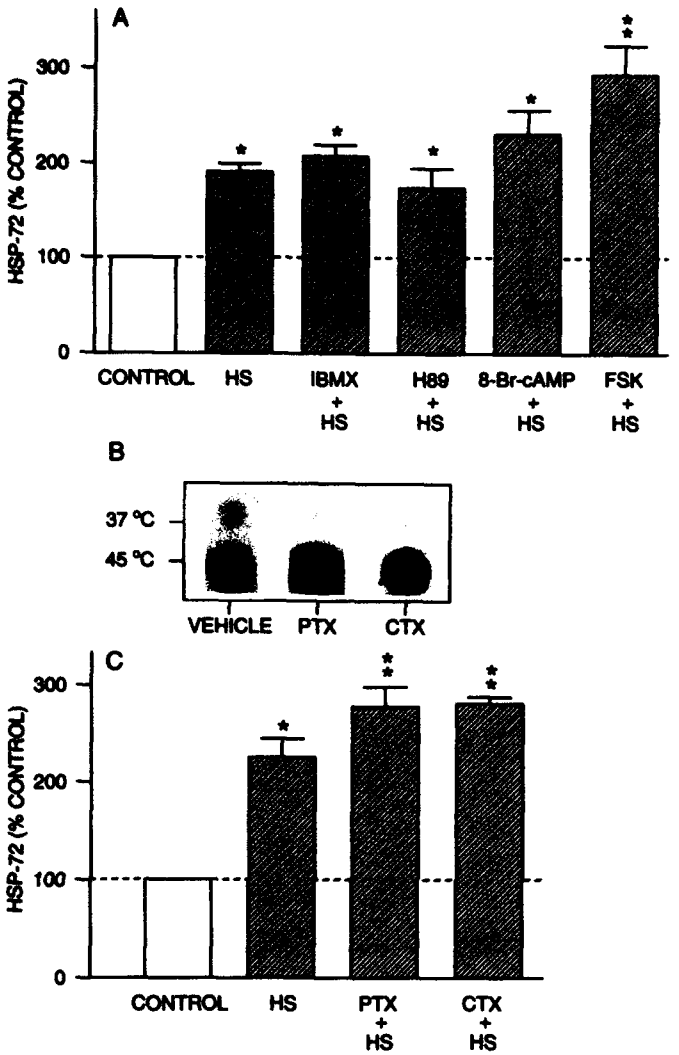

Fig. 6. Effect of 3-isobutyl-1-methylxanthine (IBMX), H-89, 8-bromo-cAMP (8-BrcAMP), forskolin (FSK), pertussis toxin (PTX), and cholera toxin (CTX) on HSP-72 induction. *A*: cells were pretreated with the following chemicals for indicated times before HS at 45°C for 10 min (n = 4–5): IBMX (1 mM, 30 min), H-89 (10 μM, 30 min), 8-BrcAMP (1 mM, 10 min), or FSK (150 μM, 10 min). These chemicals remained in buffer during heating. HSP-72 was determined 8 h after HS. $^*P < 0.05$ vs. control, $^{**}P < 0.05$ vs. control, HS, IBMX, H-89, and 8-BrcAMP, 2-way ANOVA and Bonferroni's inequality. *B*: dot blot of HSP-72 mRNA. Cells were treated with PTX (30 ng/ml, 24 h) or CTX (1 μg/ml, 1 h) before heating. HSP-72 mRNA was measured 2 h after HS (n = 3). *C*: cells were treated same as in *B*. HSP-72 was determined 8 h after HS (n = 5). $^*P < 0.05$ vs. control, $^{**}P < 0.05$ vs. control and HS, 2-way ANOVA and Studentized range test.

Effect of Ca^{2+} on the heat-induced HSP-72. Heat causes an increase in Ca^{2+} influx that is followed by a mobilization of Ca^{2+} from intracellular stores (23). This increase in $[Ca^{2+}]_i$ was dependent on external Ca^{2+} (Fig. 7*A*, see Ref. 23). If HSP-72 synthesis is triggered strictly by an increase in $[Ca^{2+}]_i$, then altering $[Ca^{2+}]_i$ without heating should increase HSP-72. We found, however, that when cells were incubated in buffers containing 10 μM A-23187 and 320, 800, or 1,600 nM Ca^{2+} for 10 min without heating, basal levels of HSP-72 did not change (data not shown). Experiments with another ionophore, ionomycin (10 μM), gave similar results. Ionomycin increased $[Ca^{2+}]_i$ from 125 ± 5 to 1,258 ± 92 nM (n = 3, $P < 0.05$), but there was no increase in HSP-72 (data not shown). These results suggest that a change in $[Ca^{2+}]_i$ without heating does not promote HSP-72 induction.

In heated cells, changes in $[Ca^{2+}]_i$ influenced the production of both HSP-72 mRNA and HSP-72. HSP-72 mRNA levels were measured in cells heated in buffers that contained different $[Ca^{2+}]$ to adjust $[Ca^{2+}]_i$ (Fig. 7*B*). Heating A-431 cells in the absence of extracellular

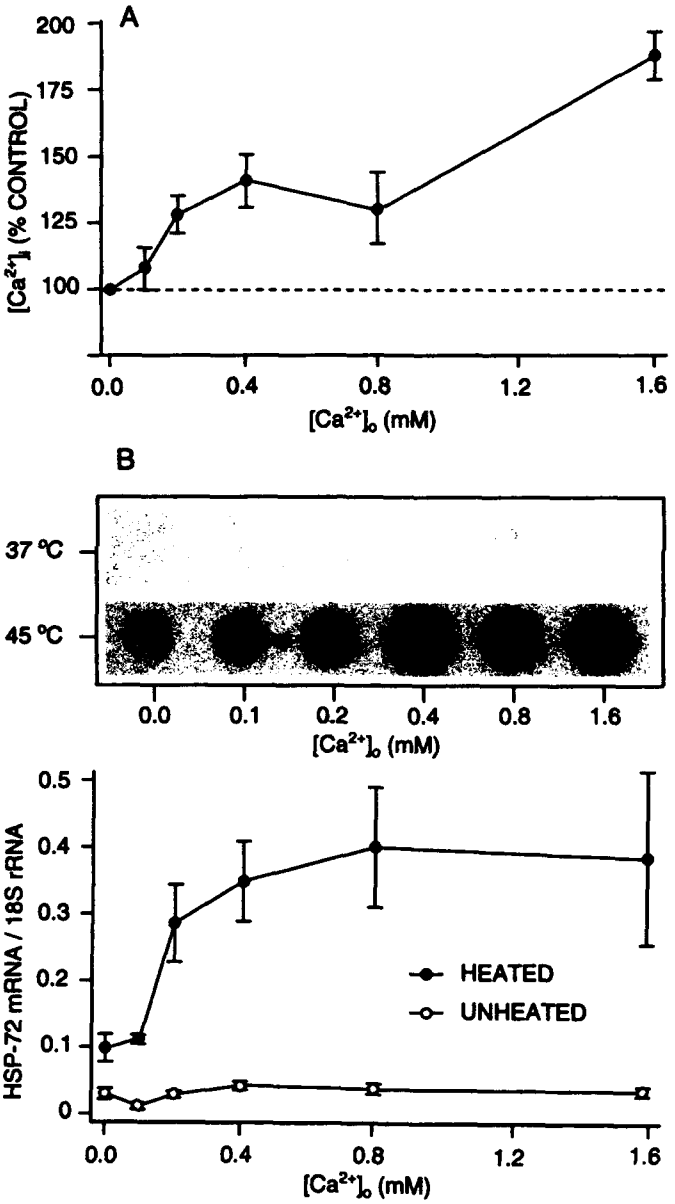

Fig. 7. Dependence of HSP-72 mRNA synthesis on Ca^{2+}. *A*: cells were exposed to heat (45°C, 10 min) in Na^+ Hanks' solution containing different Ca^{2+} concentrations ($[Ca^{2+}]$). Cells were then returned to Na^+ Hanks' solution containing 1.6 mM Ca^{2+} for 2 h before mRNA extraction and dot-blot analysis (n = 4).$[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; $[Ca^{2+}]_o$, extracellular $[Ca^{2+}]$. *B*: effect of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$. Cells were heated at 45°C for 10 min and $[Ca^{2+}]_i$ was measured immediately after heating (n = 3). Resting $[Ca^{2+}]_i$ in buffer containing 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mM Ca^{2+} were 64 ± 13, 58 ± 10, 70 ± 9, 60 ± 5, 62 ± 16, and 89 ± 16 nM (n = 3–5), respectively.

Ca^{2+} resulted in a threefold increase in HSP-72 mRNA. The addition of Ca^{2+} to the incubation resulted in a 10-fold increase, compared with unheated controls. Therefore, mRNA synthesis is apparently stimulated by the heat-induced increase in $[Ca^{2+}]_i$.

We performed other experiments that show that $[Ca^{2+}]_i$ also plays a role in enhancing the increase in HSP-72 in heated cells. Pretreatment of cells with the intracellular Ca^{2+} chelator BAPTA-AM attenuated the increase in $[Ca^{2+}]_i$ caused by heating (23), HSP-72 mRNA synthesis, and HSP-72 induction (Fig. 8*A*). Incubation of cells during heating with TMB-8, a Ca^{2+} mobilization blocker, inhibited both HSP-72 induction and increases in $[Ca^{2+}]_i$ (Fig. 8, *B* and *C*). However, it is of interest to note that an appreciable quantity of HSP-72 continued to be expressed in cells treated with BAPTA or TMB-8, which supports the view that HSP-72 induction involves both Ca^{2+}-dependent and -independent systems.

Because an increase in $[Ca^{2+}]_i$ enhanced HSP-72 mRNA synthesis and HSP-72 production only after heating, we sought to determine whether Ca^{2+} might be playing a role in the binding of the heat-shock transcriptional factor (HSF) to the HSE. Electrophoretic mobility shift assays show that both slow- and fast-moving HSF-HSE complexes increased 331 and 346%, respectively, after heating in the presence of external Ca^{2+} (Fig. 9, *lane 3*). The fast-moving complex consists of HSE bound to an HSF trimer, while the slow-moving complex is thought to consist of HSE bound to an HSF hexamer (29). The HSE binding was not artifactual because no binding was observed in the presence of a 50× excess of unlabeled HSE (Fig. 9, *lane 6*). The amount of both complexes was reduced markedly in cells heated in the absence of external Ca^{2+} or in cells treated with 100 μM BAPTA (Fig. 9, *lanes 8* and *9*).

DISCUSSION

This study demonstrates that heat shock induced the synthesis of a temperature-dependent HSP-72. Maximal HSP-72 mRNA synthesis and HSP-72 induction occurred 2 and 8 h, respectively, after heat shock. Elevated levels of HSP-72 continued in A-431 cells for at least 4 days after heating. In general, the length of time that heat-shock proteins persist after heating varies with the kinds of cells and tissues examined. It remains detectable in rat brain for 4 days after heating (9) but can be detected in heart, lung, liver, spleen, adrenals, and bladder for up to 16 days (9). HSP remains for 8 days in rat heart muscle (21) and persists for 10 days in rat kidney (14). However, increases in HSP appear to be more transient in human monocytes (40).

The increase in HSP-72 occurs as a result of an increase in the synthesis of its mRNA, a process that can be inhibited by actinomycin D. Maximal synthesis of HSP-72 mRNA occurred 2 h after heating and returned to baseline levels within 8 h. This is consistent with studies of human monocytes (40) and rat skeletal muscle, heart, and liver (44). Other studies measured elevated levels of HSP-72 mRNA in rabbit heart (28) and *Mongolia gerblis* (38) for 24 h and 3–4 days, respectively. The

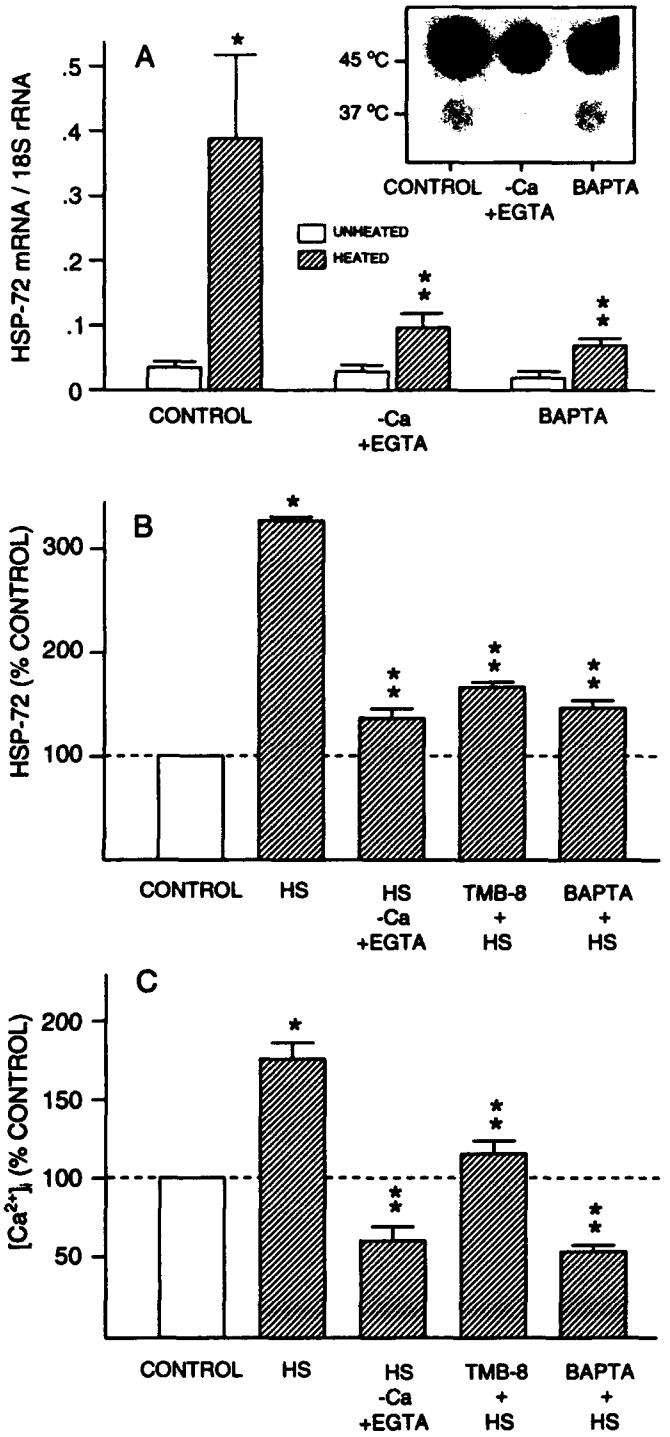

Fig. 8. Effect of the heat-induced increase in $[Ca^{2+}]_i$ on HSP-72 mRNA and HSP-72. Cells were exposed to heat (45°C, 10 min) in absence of Ca^{2+} with 10 mM EGTA, in presence of 100 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-acetoxymethyl ester (AM), or 100 μM 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8). Cells were returned to 37°C for 2 h before mRNA extraction (*A*) or 8 h before HSP-72 determination (*B*; n = 3–5). *A*, *inset*: dot-blot of *A*. *C*: $[Ca^{2+}]_i$ was measured immediately after heating (n = 3–9). *$P < 0.05$ vs. controls and all other treatments; **$P < 0.05$ vs. controls and HS, determined by one-way ANOVA and Bonferroni's inequality.

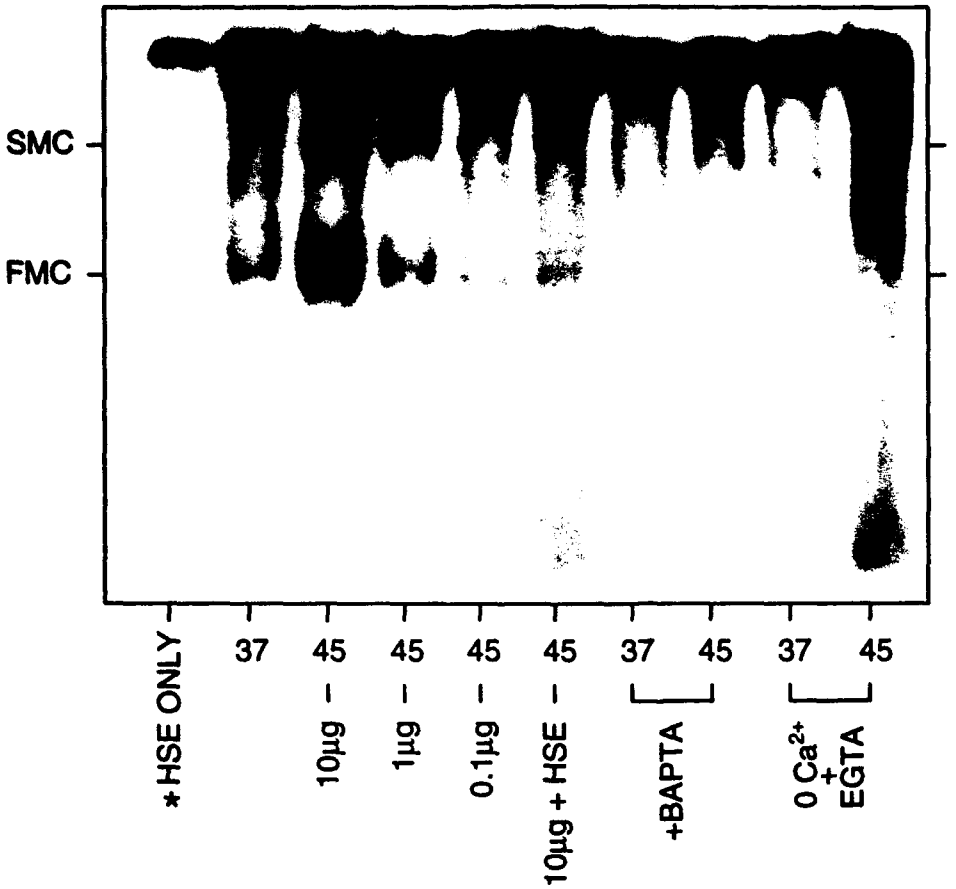

Fig. 9. Effect of inhibiting the heat-induced increase in $[Ca^{2+}]_i$ on heat-shock transcription factor (HSF)-heat-shock element (HSE) complex formation. Cells were exposed to heat (45°C, 10 min) in presence of 1.6 mM Ca^{2+} without BAPTA (*lanes 3-6*) or with BAPTA (100 μM, 10 min, *lanes 7* and *8*) or in medium containing 10 mM EGTA without Ca^{2+} (*lanes 9* and *10*). Nuclear protein was extracted and incubated with radiolabeled HSE to detect HSF. *Lane 1*, ^{32}P-labeled HSE (*HSE) with no nuclear protein; *lane 2*, unheated control (37°C), 10-μg nuclear protein; *lanes 3-5*, heated (45°C) with indicated amount of nuclear protein; *lane 6*, heated (45°C), 10 μg of nuclear protein with 50× excess of unlabeled HSE to compete with *HSE; *lanes 7* and *8*, 10-μg nuclear protein extracted from unheated and heated cells treated with BAPTA; *lanes 9* and *10*, 10 μg of nuclear protein extracted from cells not heated and heated, respectively, in buffer containing 0 Ca^{2+} and 10 mM EGTA. SMC, slow-moving complex; FMC, fast-moving complex.

difference between the short-lived mRNA found in A-431 cells and the long-lived mRNA in rabbit heart and *Mongolia gerblis* may reflect species differences and/or the type of stimulation.

In the present study, the amount of HSP-72 in heated cells remained greater than in unheated cells even after mRNA returned to baseline levels. This is apparently due to the rather long half-life of HSP-72 and not to any direct effect of heat on protein translation. This conclusion is supported by our data, which show that cells treated with cycloheximide 8 h after heating had levels of HSP-72 synthesis similar to cells not treated with cycloheximide. Also, heated cells demonstrated no greater [^{35}S]methionine incorporation into HSP-72 than unheated controls after HSP-72 mRNA levels had returned to the baseline. The relatively long half-life of the HSP-70 family of proteins has been used to explain why they remain elevated for extended periods in certain tissues and cells (9, 14, 21).

We performed experiments in which A-431 cells were heated a second time after the first heating in an effort to increase the levels of HSP-72 synthesis. The second heating led to no further increase in the amount of HSP-72 present in the cell. This interesting observation suggests that the initial exposure to heat induces changes in the cell that alter normal heat-response pathways, making them unavailable at the time of a second heating. This might also explain why cells heated to 45°C for more than 10 min did not produce additional HSP-72. Although further study is required, it is possible that these changes relate to alterations in Ca^{2+} metabolism that occur in thermotolerant cells (25).

The induction of HSP-72 by heat is not associated with an intracellular acidification or an increase in cAMP. These results are in agreement with the finding of Drummond et al. (13), which showed that intracellular acidification in *Drosophila* salivary glands does not induce HSP. However, Weitzel et al. (55) correlated a significant increase in the synthesis rate of heat-shock proteins in yeast with a decrease of pH_i. It has also been reported that the activation of many heat-shock genes in yeast is caused by a decline in intracellular cAMP (39). The differences between our data and those from yeast may be the result of the different cells used in the experiments.

It appears that G proteins are involved in the induction of HSP-72 by heat. PTX, CTX, and FSK (by virtue of its ability to stimulate G proteins) potentiated heat-induced HSP-72. Previously, this laboratory reported that these agents increase inositol trisphosphates (24) and that CTX and FSK, but not PTX, increase cellular cAMP (27). It is therefore likely that PTX, CTX, and FSK potentiate HSP-72 by increasing inositol trisphosphates. This is supported by the observation that U-73122 (an inhibitor of inositol trisphosphates production) markedly inhibited HSP-72 induction. It has been found that binding of inositol trisphosphate to its receptor alters RNA splicing (10, 34, 36) and regulates

multiple gene products (3, 52). It remains unclear whether this small but significant enhancement of HSP-72 induced by treatment with PTX, CTX, or FSK is a result of a direct activation of G proteins or the production of inositol trisphosphates. Further tests of the proposed role of G-proteins and inositol trisphosphates and the functional significance of the enhancement of HSP-72 are in progress.

The induction of HSP-72 and its mRNA by heat depends on the heat-induced increase in $[Ca^{2+}]_i$. The synthesis of both HSP-72 mRNA and HSP-72 demonstrated both Ca^{2+}-dependent and -independent aspects. The existence of Ca^{2+}-dependent processes is demonstrated by three observations. *1*) If no Ca^{2+} is added to the incubation buffer during heating, thereby providing no Ca^{2+} for the heat-induced Ca^{2+} influx, mRNA and HSP-72 increased 300 and 45%, respectively, after heating. But if 1.6 mM Ca^{2+} is added to the incubation buffer during heating, to provide Ca^{2+} for the heat-induced Ca^{2+} influx, there was a significantly larger increase in the amounts of mRNA and HSP-72 (13-fold and 239 ± 17 %, respectively). *2*) Treatment with BAPTA-AM (which, on conversion to BAPTA inside the cell, chelates the heat-induced increase in free Ca^{2+} in the cytosol) significantly reduced mRNA and HSP-72 synthesis after heating. *3*) Treatment with TMB-8 (which inhibits the mobilization of intracellular Ca^{2+} stimulated by the heat-induced influx of extracellular Ca^{2+}) diminished the heat-induced induction of HSP-72. Other reports have also shown what appears to be a variable role for Ca^{2+} in heat-shock protein induction. HSP-26 in mouse mammary tumor C127 cells (15) depends on Ca^{2+}, but increases in $[Ca^{2+}]_i$ in human monocytes (20), *Drosophila* salivary gland (13), and rat GH_3 pituitary cells (5) are thought not to be correlated with heat-induced heat-shock protein synthesis.

The exact role of $[Ca^{2+}]_i$ remains somewhat unclear, however, because our data also show that adjusting $[Ca^{2+}]_i$ to a level similar to that observed after heat shock failed to induce HSP-72. It is possible that the increase in $[Ca^{2+}]_i$ that occurs after heating serves to enhance, but not necessarily trigger, HSP-72 synthesis.

Evidence is accumulating that a change in the environment of the cell, such as heating, denatures certain proteins, which permits the activation of a preexisting HSF residing in the cytosol (16). The activated HSF forms a trimer that enters the nucleus to bind to the HSE located on the heat-shock gene. This binding then triggers the initiation of transcription (see reviews in Refs. 46, 50, and 58). The processes of HSF activation and HSF-HSE binding can be induced by Ca^{2+}. Mosser et al. (35) demonstrated that HSF activates and binds to HSE in the presence of either whole cell or cytoplasmic extracts from HeLa cells in the presence of Ca^{2+}. Also, Price and Calderwood (42) reported that both Ca^{2+} and ATP are required in digitonin-permeabilized National Institutes of Health 3T3 cells before transcription of the HSP gene. Our preliminary data with nuclear extract from A-431 cells show that heating, under conditions where $[Ca^{2+}]_i$ was allowed to increase, led to a greater degree of HSF-HSE binding complex formation than that observed when the heat-induced increase in $[Ca^{2+}]_i$ was blocked. It is possible that an increase in $[Ca^{2+}]_i$ is critical for translocation of HSF from cytoplasm to nucleus, and more HSF is available for HSE. It is interesting to note that the transcription process for endothelin-induced (41) and corticotropin-releasing factor-induced c-*fos* and c-*jun* mRNA (4) is also Ca^{2+} dependent. A detailed mechanism for this process in A-431 cells remains to be elucidated.

In summary, heat shock induced a temperature-dependent HSP-72 that was related to the duration of heating. The increase resulted from a heat-induced stimulation of transcription, and the high levels of induced HSP-72 were maintained because of the long half-life of the protein in the cytoplasm. The increase in HSP-72, its mRNA, and HSF-HSE-binding complexes occurred independently from an increase in $[Ca^{2+}]_i$ but was enhanced by an increase in $[Ca^{2+}]_i$. PTX- and CTX-sensitive G proteins also potentiated the synthesis of heat-induced HSP-72 mRNA and HSP-72. It appears that Ca^{2+} and G proteins play roles as enhancers of HSP-72 synthesis induced by heat shock.

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